Preparation and Characterization of Bioplastic film by Deoiled cake and Polyhydroxybutyrate producing bacteria isolated from diverse sources

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Synopsis

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Introduction :

We are using plastic in vast amounts in our daily life. Indiscriminate use of plastics has led to increased pollution in the environment and various diseases are spreading in the atmosphere. We are using the plastic almost in every material of our daily use. For example-disposable cups, plastic glasses, water bottles, milk jugs, plastic bags to carry groceries, knives, folks, coffee cups, utensils, pipes, straw etc.

Petroleum based plastic is non renewable and not eco friendly. If we want to reduce the use of plastic on a large scale we have to produce a proper replacement.

The major problem of using plastic is that it can't be degraded because it is made up of fossil fuel .That's why its mass is collected in a huge amount across the road, in the river, in ponds, plains etc. and causes various diseases. Plastic fulfills our needs but at the end of the day it returns disease with pollution to us. Need of the hour is to produce a biodegradable replacement of plastic.

A biodegradable or eco-friendly substitute will not cause disease and pollution. We can use the bioplastic to get rid of plastic. Bioplastic are the long chain of monomers that are manufactured into the commercial product from natural sources or renewable sources like starch, cellulose, proteins etc.

Three generations of bioplastic are in use. These are as follows-

In the first generation of bioplastic; corn, rice, wheat, sugarcane, potato, sugar beet and plant oil were used.

In the second generation of bioplastic; wood, short- rotation crops such as palm, wheat, straw were used.

Third generation feedstock included algae or seaweed, bacteria and some fungi.

A purely sustainable and renewable product can cure the problem of plastic. Agricultural waste is one of them. Agricultural waste involves cotton linters, jute fibers, potato peel, rice straw, wheat straw, de-oiled cake of mustard, soya bean and ground nut etc. Bioplastic from agricultural waste is much better than petroleum based plastic.

Properties	**Petroleum based plastic**	**Bioplastic**
• **Renewable**	No	Yes or partially
• **Sustainable**	No	Yes
• **Polymer range**	Wide ranging	Limited biopolymers (lipids, proteins, starch etc), bacterial

		polymers (poly hydroxy alkanoates, PHA, and polyhydroxy butyrate, PHB)
• **Discontinuity in the environment**	Usually non degradable or some degradation by polymer oxidation	Biodegradable and/or compostable
• **Greenhouse gas emission**	High	Low
• **Utilization of fossil fuels**	High	Low
• **Agriculture land utilization**	No utilization	Predict expansion

(Bioplastics www. hgea. com)

Review of literature:

International reports :

- Bilo. F. et. al, 2018 reported that the Naviglio extractor was employed for the first time to pretreat rice straw. Cellulose-based bioplastic possesses very good mechanical features, with tensile strength and elongation at break over 43 MPa and nearly 6% for dried dumbbells and 11 MPa and 63% for wet samples, respectively. After extraction, the co-solubilization of cellulose of solid matrix with other organic matter in rice straw was done by the treatment only with TFA.
- Getachew A and Woldesenbet F., 2016 examined fifty bacterial strains isolated from different localities, and selected ten PHB accumulating strains and compared them for their ability to accumulate PHB granules inside their cells. *Bacillus* spp. was found to be the best producer. The production cost was reduced by using agro-residues, especially sugarcane bagasse for feasible production of PHB, using isolated *Bacillus spp*.
- Mohamed samer et. al., 2019 considered that it is possible to produce bio-based plastics from starch extracted from potato peels. The flexibility of the bioplastic material increased using glycerin, as it makes the chains of starch slip along with each other.
- E. B. Arikan and D. Bilgen, 2019 concluded that food wastes could be used for bioplastic production. In this study, it was determined that the bioplastics produced from potato peels completely degraded within 28 days, and it was suggested that these bioplastics can be used in the packaging industry. On the other hand, it was observed that the commercial bioplastic did not biodegrade in 28 days.
- Occurrence of PHAs in bacteria has been known since the 1920s, when Lemoigne noted the

structure of poly 3 hydroxybutyrate inside bacterial cells. (Lemoigne 1926). PHAs are environmentally friendly, biocompatible and bio- degradable thermoplastics. Renewable vegetable oils and various waste products used as alternative substrates. Novel extraction methods, genetically enhanced species and mixed cultures with a view to make PHAs more commercially attractive were suggested by R.A.J. Verlinden et. al.,2007.

- F.Karim et. al., 2018 investigated and screened sixteen potential poly-β-hydroxybutyrate (PHB) producing bacteria using Nile red and Sudan black. Acridine orange was used to visualize PHB granules under a fluorescent microscope. By analyzing photomicrographs of 24 hour old cultures, 6 out of 16 isolates showed good PHB accumulation capacity. These isolates belonged to four genera viz. *Bacillus, Pseudomonas*, *Micrococcus* and *Veillonella*. These bacteria can be a potential source for commercial production of biopolymer and can also be tested for high PHB yield using other renewable substrates.
- Thapa. C. et al.,2018 examined that *B. arthrobacter* and *Micrococcus sp*. produced PHB in good quantity among other isolated species. and the production of PHB from fermentation methods and broth culture gave much better results rather than that from agar in all the isolated species.

National reports :

- S. Kumaravel et. al., 2010 suggested that PHB produced from sugarcane effluent can be degraded by microorganisms, present in the garden soil. To get rid of the hazardous problems, these industrial effluents could be utilized in an effective way and can be a boon for agriculturalists and industrialists. These industrial effluents can be used as substrate for PHB (bioplastic) production and this bioplastic can be degraded by microorganisms without use of any chemical. Fungal isolates degraded the PHB better than bacteria.
- Mukesh chander 2019 investigated that raw material such as cheese whey, cassava starch, banana peels, corn, potato, wheat straw etc. can be used for the production of biodegradable plastic. All these substrates had the ability to make bioplastic successfully using different methods. At industrial levels, two bacterial strains have been mainly used for the production of bulk biodegradable plastic. These strains are namely the *Bacillus licheniformis* and *Bacillus megaterium*.

- M.K. Marichelvam et. al., 2019 revealed that the corn and rice starches have better biodegradability than the existing plastic materials. Addition of citric acid improved the shelf-life of the material and mechanical properties of bioplastic. Bioplastics can be used as packing materials and can be used as an alternative to LDPE and HDPE plastic bags. Due to the obtained properties of bioplastic, it would be interesting to prepare polybags using this bioplastic with assumed lower cost.

Objectives:

- **Screening and preservation of PHB producing bacteria from compost and soil from the automobile washing area.**
- **Identification of the highest PHB producing bacterial isolates.**
- **PHB production using broth culture and its recovery for bioplastic preparation.**
- **Isolation of protein from de-oiled cake of mustard and sunflower.**
- **Preparation, characterization and comparison of bioplastic film from isolated protein and PHB granules.**
- **Biodegradability test of prepared bioplastic.**

Plan of action:

1. Screening and preservation of PHB producing bacteria from compost and soil from the automobile washing area:-

Collection of soil sample and compost will be done from selected areas of Agra.

- **Isolation of bacteria:** Bacteria will be isolated from soil sample and compost and will be grown in nutrient agar medium via serial dilution technique.
- **Identification of bacteria:** Isolated bacterial strains will be identified on the basis of microscopic, macroscopic and biochemical tests. Bacterial strains will be identified by examining its size, colony, morphology, surface pigment, motility test and gram staining.
- **Screening of PHB producing bacteria:** There is no such standard media available to isolate PHB producing bacteria. Therefore to isolate PHB producing bacteria different types of media will be prepared. i.e. Whey agar medium (Khanafari et al., 2006), PHA production medium (Yan et al.,2005), mineral salt medium (Halami, 2008), solid HM

medium (Quillaguman et al., 2005), yeast extract mannitol agar (YEMA) (Deshwal and Chaubey,2014). Only PHB producing bacteria will show colonies on these media otherwise no colonies will be observed. Different dyes will be used to observe characteristics of PHB producing bacteria.

- **Preservation of microorganisms:** isolated bacterial slants will be stored at 4˚C in NAM media and will be revived once in a month.

2. Identification of the highest PHB producing bacterial isolates:-

PHB producing ability of isolated bacterial strain will be determined by primary screening i.e. plate assay and secondary screening (submerged fermentation). Higher PHB producing strain will be used for further studies.

2.1) DNA isolation and molecular identification: molecular identification of selected strain will be done by different techniques:

- **Isolation of DNA**
- **Amplification by PCR**
- **Gene sequencing**

3. PHB production using broth culture and its recovery for bioplastic preparation.

3.1) PHB production in broth culture: Old culture will be inoculated in nutrient broth and YEM media. It will be used as an inoculum for each micro-organism. After 24 hrs of growing 500ml of respective media will be added to it. Flask will be incubated inside the shaking incubator at 120 rpm/min for 48-72 hrs at 37˚C (Chauhan et al., 2013).

3.2) PHB recovery using chloroform: Hot acetone will be used for washing of cells for 20min. Once dried the cells will be mixed with 50 volumes of chloroform, for 48 hrs at 30˚C. Centrifugation will be done for the recovery of clear polymer solution. Polishing filtration method will be followed to remove majority of non PHB material. Non-solvent precipitation (5 times the volume of chloroform) and filtration method will be finally used to obtain pure PHB. Mixtures of methanol and water will be used as non-solvent (Chauhan et al., 2013).

4. Isolation of protein from de-oiled cake of mustard and sunflower:-

The de-oiled cake will be collected from de-oiled cake manufacturers, Agra.

4.1) Chemical analysis of de-oiled cake: Chemical analysis of de- oiled cake will be done by following methods-

4.1.1) Protein content: Kjeldahl method is a standard method of the Association Of Official Analytical Chemist (AOAC,2012) ,and will be used to measure protein content of mustard de-oiled cake.

4.1.2) Oil content : Soxhlet apparatus is a standard method of the Association Of Official Analytical Chemists (AOAC, 1990) to measure oil content of mustard de-oiled cake.

4.2) Extraction of protein of de-oiled cake of mustard: Alkali soluble and acid precipitation will be used for protein extraction from de-oiled cake. Folin-lowry method will be used to determine the concentration of protein.

5. Preparation of bioplastic films from isolated protein and PHB granules and its characterization and comparison:-

Protein isolates, produced PHB granules, polyvinyl alcohol (PVA) and other additives will be used for preparation of bioplastic films. Formulation of bioplastic will be done by solution casting method (Hewage S. and Vithanarachchi S. 2009).

Characterization and comparison of bioplastic: Prepared film will be analyzed by- FTIR(Fourier-transform infrared spectroscopy)

XRD(X-ray diffraction)

Comparison of both prepared bioplastic films will be carried out by mechanical properties.

6. Biodegradability test of prepared bioplastic:-

6.1) Biodegradation test of bio-plastic film in natural conditions: The soil burial test will be used to check out the biodegradability of bioplastic.

6.2) Biodegradation test of bio-plastic sheets in laboratory conditions:

6.2.1) Biodegradation by composting: The weight of bioplastic will be measured first. Prepared bioplastic will be buried with the municipal solid waste mixture. After this possible biodegradation of bioplastic will be measured. The paper waste, leaves, cow manure, food waste, and water are included in the municipal solid waste mixture inside the oven at 55˚C. The maximum growth of thermophilic microorganisms will occur at this temperature. The

sample will be weighed every third day and percentage of weight loss will be examined.

6.2.2) Biodegradation through shake flask experiment or liquid culture method: Pre-weighed bioplastic will be transferred aseptically into a conical flask containing 300ml mineral salt medium (broth) and will be inoculated with already identified polythene degrading microorganisms. In the microbe free medium the control will be kept in a rotary shaker at 30˚C, 150rpm for 1,2 & 3 months. After shaking we will collect the bioplastic and wash it with distilled water, dried in the shade, and it will be analyzed for biodeterioration.

6.3) Effect of degradation on bioplastic: Effect of degradation on bioplastic will be carried out through-

- **Weight reduction**
- **CO2 analysis**
- **FTIR analysis**

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